SET1 PROTEINS AND USES THEREFOR

FIELD

The present invention relates to methods of use of SET1 (also known as SSL7) proteins or

5 functional equivalents thereof. More particularly the invention relates to the use of SET1 proteins or functional equivalents thereof in procedures for identification and/or isolation of IgA and the serum complement factor C5.

BACKGROUND

10 The gram-positive bacterium *Staphylococcus aureus* is a common human pathogen causing food poisoning, skin infections, abscesses, bacteraemia, pneumonia, endocarditis, osteomyelitis, toxic shock syndrome, staphylococcal scarlet fever and scalded skin syndrome. *S. aureus* produces a plethora of cell-surface and secreted proteins that bind host cell-surface receptors, extracellular matrix proteins, and soluble serum factors

15 involved in both innate and adaptive immunity (1). Several studies (2) and the recent completion of several staphylococcal genomes has revealed that many of these virulence factors are clustered within 3 distinct pathogenicity islands SaPIn1, SaPIn2 and SaPIn3 (3, 4). One important class of genes within SaPIn1 and SaPIn3 are those that code for superantigens. These potent exotoxins target the antigen recognition receptors of the

20 adaptive immune response - the Major Histocompatibility Complex class II (MHC-II) and T cell Receptor (TcR) and drive antigen independent T-cell activation and cytokine release (5) possibly in an attempt to prevent local inflammation and leukocyte recruitment to the site of infection through the production of T cell cytokines (4).

25 The staphylococcal exotoxin-like proteins (SETs) encoded by genes clustered within the staphylococcal pathogenicity island SaPIn2 are superantigen homologues (8). Comparative alignment of SETs and superantigen sequences indicate that they have evolved from the same ancestral gene (3) and are most similar to toxic shock syndrome toxin 1 (TSST-1) whose gene resides on SaPIn1. TSST-1 is a TcR Vβ2 binding

30 superantigen produced by toxigenic strains of *S. aureus* associated with Toxic Shock Syndrome (9). Twenty-six members of the SET family have been identified (8) (3) (10),

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although several appear to be allelic variants. For example SET1 from strain NCTC6571 (8), SET11 from N315 and Mu50 (3), and SET22 from MW2 (10) are probably the same protein. The 3-D structure of SET3 has been determined and displays the same OB-fold/β-grasp two-domain structure as the superantigen (11) with conservation of the central core region at the interface of the two domains. Yet the SETs are not superantigens. They do not bind MHC class II nor activate T cells. Those regions on SET3 corresponding to the MHC class II or TcR binding sites on superantigens are substantially altered (11). The function of SETs remains unknown although the presence of their genes on SaPIn2 may indicate that they are part of the bacterial defense armamentarium (11) (8) (12). Notably an *set15⁻* mutant of *S. aureus* displayed a 30-fold reduction in bacterial persistence in a murine kidney abscess infection model.

SET proteins may also be known as staphylococcal superantigen-like proteins (or SSLs). There has been a move recently to use SSL when naming these proteins.

IgA is the predominant antibody located at mucosal surfaces and the second most predominant isotype in serum. In humans the IgA production of approximately 66mg/kg/day exceeds that of all other Ig classes combined (13). Mucosal IgA exists mostly as a dimer complexed with a J-chain and a secretory component (SC). Polymeric IgA binds to the polyIg receptor on the basolateral surface of the mucosal epithelium to be transcytosed through the epithelial layer and released into the mucosa as a covalent complex called secretory IgA (sIgA). The cleaved ectodomain of the pIgR remains bound as the secretory component (SC). Secretory IgA is unable to bind FcαR and activate phagocytosis in the absence of an integrin co-factor Mac-1 (complement receptor 3 or CD11b) (17). (Reviewed in (15), (16)). Serum IgA on the other hand is predominantly monomeric and only binds avidly to its receptor FcαRI (CD89) on formation of immune complexes. Binding and cross-linking of FcαRI (CD89) by IgA immune complexes initiates phagocytosis by neutrophils, granulocytes and monocyte/macrophages (17). Monomeric serum IgA has been proposed to provide a second line of defense against microbes such as S. aureus via FcαRI mediated phagocytosis (18, 19). Mutagenesis (20-

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22) and the recent crystal structure of an IgA:FcαR complex reveals that the FcαR Ig-like D1 domain binds at the Cα2:Cα3 junction of the IgA H-chain(15).

Complement C5 is the central component in the terminal stage of the classical, alternative, and lectin mediated complement pathways. Complement C5 is ~189 kD and is synthesised as an intracellular single-chain precursor that is secreted as a two-chain glycoprotein consisting of a 75 kD N-terminal C5β fragment disulfide linked to a 115 kD C-terminal C5α fragment ((23, 24)). The surface bound C5 convertases generated from either the classical, alternative or lectin pathway; cleave soluble C5 to generate two active fragments C5a and C5b. The potent anaphylatoxin C5a is a 74-residue N-terminus fragment cleaved from C5α by C5 convertase. C5a binds a G-protein coupled receptor C5aR on the surface of myeloid cells to stimulate a range of pro-inflammatory and chemotactic actions such as oxidative burst, phagocytosis and leukocyte recruitment which all contribute to the defense against organisms such as *S. aureus* (25). The C5b fragment initiates assembly of the terminal complement components into the membrane attack complex (MAC) that forms a water permeable membrane channel leading to cell lysis.

Methods are known for the purification of immunoglobulins, including IgA. For example, purification may be achieved by methods including classical protein purification techniques, such as ion exchange and size exclusion chromatography, and by affinity purification using a number of bacterial derived proteins, such as Protein L. These techniques may suffer from a number of problems including being laborious, lacking specificity and can result in low yields and purity. For example, Protein L, derived from the bacterium *Peptostreptococcus magnus,* and which is used for purification of immunoglobulins, binds to a wide variety of immunoglobulins. This means that it may not be suitable for the purification of a single species of immunoglobulin from a heterogenous mix of immunoglobulins.

An example of a product which may allow for single step purification of IgA and IgE is the affinity purification product known as Kaptiv-ae™ (Genomics One International Inc,

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USA). This is a synthetic peptidomimetic compound that selectively binds IgA and IgE from several species.

Single step purification methods for complement C5 are not known. Current methods to purify complement C5 from human serum, for example, rely on multiple chromatographic steps such as ion exchange and size exclusion chromatography. These may often result in low yields of final product.

Accordingly, there may be considered a need to provide an alternative or improved method of isolating and identifying IgA and C5.

Bibliographic details of the publications referred to herein are collected at the end of the description.

OBJECT

It is an object of the present invention to provide an improved method of removing, isolating and/or detecting IgA and/or C5 or at least to provide the public with a useful choice.

STATEMENT OF INVENTION

In accordance with the present invention it has been discovered that recombinant SET1 binds independently IgA and the serum complement factor C5. This may have significant application in methods of isolating or removing IgA and C5 from samples and also in identification and quantitation of IgA and C5 in samples for diagnostic purposes, for example.

In a first aspect of the invention there is provided a method of isolating IgA present in a sample, the method comprising at least the steps of:

Bringing SET1 or a functional equivalent thereof in contact with the sample for a period sufficient to allow SET1 or functional equivalent thereof to bind to IgA to form a complex;

Separating the complex; and

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Releasing IgA from the complex.

In another aspect of the invention there is provided a method of isolating C5 present in a sample, the method comprising at least the steps of:

5   Bringing SET1 or a functional equivalent thereof in contact with the sample for a period sufficient to allow SET1 or functional equivalent thereof to bind to C5 to form a complex; Separating the complex; and Releasing C5 from the complex.

10   In a preferred aspect of the present invention there is provided a method for isolating IgA from a sample, the method comprising at least the steps of:
Providing a matrix to which a SET1 protein or functional equivalent thereof is bound; Providing a sample; Bringing said matrix and said sample into contact for a period sufficient to allow SET1 or
15   functional equivalent thereof to bind to IgA present in the sample; and, Releasing IgA from the matrix.

Preferably, the method further comprises the step of collecting the IgA released.

20   Preferably, the matrix is in the form of a column over which the sample is passed.

Preferably the method further comprises the step of washing contaminants present in the sample from the matrix prior to release of IgA.

25   Preferably the matrix is Sepharose.

Preferably the sample is milk or colostrum. More preferably the sample is serum.

Preferably the method further comprises the step of determining the quantity of IgA
30   present in the sample.

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Preferably, IgA is released from the matrix using a 100mM glycine buffer at pH 3.0.

In a preferred aspect of the present invention there is provided a method for isolating C5 from a sample, the method comprising at least the steps of:

5   Providing a matrix to which a SET1 protein or functional equivalent thereof is bound;
Providing a sample;
Bringing said matrix and said sample into contact for a period sufficient to allow SET1 or functional equivalent thereof to bind to C5 present in the sample; and,
Releasing C5 from the matrix.

10

Preferably, the method further comprises the step of collecting the C5 released.

Preferably, the matrix is in the form of a column over which the sample is passed.

15   Preferably the method further comprises the step of washing contaminants present in the sample from the matrix prior to release of C5.

Preferably the matrix is Sepharose.

20   Preferably the sample is milk or colostrum. More preferably the sample is serum.

Preferably the method further comprises the step of determining the quantity of C5 present in the sample.

25   Preferably, C5 is released low pH buffer such as 50mM acetate pH 3.5.

In another aspect the invention provides a method of detecting IgA and/or C5 in a sample, the method comprising at least the steps of:
Contacting a sample with SET1 or functional equivalent thereof for a period sufficient to
30   allow SET1 or functional equivalent thereof to bind to IgA and/or C5; and,
Detecting bound SET1 or functional equivalent thereof.

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Preferably, the method further includes the step of determining or quantifying the level of bound SET1.

Preferably, the method is conducted for the purpose of diagnosing IgA and/or C5
5   abnormalities in a subject.

Preferably the subject is a mammal, more preferably a human.

In another aspect of the invention there is provided a method of removing IgA from a
10   sample, the method comprising at least the steps of:
Bringing SET1 or a functional equivalent thereof in contact with the sample for a period sufficient to allow SET1 or functional equivalent thereof to bind to IgA to form a complex;
Separating the complex from the sample.

15   In another aspect of the invention there is provided a method of removing C5 from a ·
sample, the method comprising at least the steps of:
Bringing SET1 or a functional equivalent thereof in contact with the sample for a period sufficient to allow SET1 or functional equivalent thereof to bind to C5 to form a complex;
Separating the complex from the sample.
20
In another aspect, the invention provides a kit for the detection of IgA and/or C5 in a sample, the kit comprising at least SET1 or a functional equivalent thereof.

In a further aspect, the invention provides a kit for the isolation of IgA and/or C5 from a
25   sample, the kit comprising at least SET1 or a functional equivalent thereof.

In another aspect, the invention provides a kit for the removal of IgA and/or C5 from a sample, the kit comprising at least SET1 or a functional equivalent thereof.

30   In a further aspect, the present invention provides a novel·SET1 protein having the amino acid sequence SEQ ID NO: 6 or SEQ ID NO: 7.

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In a related broad aspect, the invention provides a nucleic acid encoding a SET1 protein of SEQ ID NO:6 or SEQ ID NO:7.

5   Preferably, the nucleic acid sequence is that represented by SEQ ID NO:12 or SEQ ID NO:13.

The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively,

10   in any or all combinations of two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which the invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

15   **FIGURES**

These and other aspects of the present invention, which should be considered in all its novel aspects, will become apparent from the following description, which is given by way of example only, with reference to the accompanying figures, in which:

20   **Figure 1.** Illustrates Purified recombinant proteins.   10 μg of recombinant FcαRI ectodomain produced in baculovirus (22) (lane 2) and 10 μg of SET1 produced in E. coli (lane 3) were analyzed by SDS PAGE under reducing conditions. Samples of purified human polyclonal IgA were also run for size comparison.

25   **Figure 2.** Illustrates Serum proteins bound by SET1. A. Recombinant SET1 coupled to sepharose purified proteins from human serum (lane 3) but not from fetal calf serum (lane 2) of PBS (lane 1) controls. B. Comparison of protein A (lane 1) and SET1 purified Ig (lane 2) showing that the heavy chain for protein A purified IgG is 50 kD while that for SSL7 was 60 kD. C. Proteins purified

30   from human serum by either protein A (lane 1) or SET1 (lane 2) were analyzed by Western blotting using human isotype specific anti-sera. Only the anti-IgA

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anti-serum (panel 1) detected the SSL7 bound IgA heavy chain at 60 kD. Anti-IgD, IgG and IgM did not react to SET1 purified serum proteins. All SDS PAGE gels were run under reducing conditions.

5   **Figure 3.** Illustrates SET1 binds IgA and C5 from multiple sites, individuals and species. A. SET1 sepharose purified proteins from serum (10μl), breast milk (10 μl), saliva (100 μl), and tears (100 μl), and analyzed by SDS PAGE under reducing conditions. The arrow indicates the position of the secretory component (SC) associated with secretory IgA observed in breast milk, saliva and tear but absent
10   from serum. The serum sample contains the additional C5 alpha and C5 beta chains that are absent in the breast milk, saliva and tears. **B.** SET1 purified proteins from the serum of 14 human volunteers. 10 μl of serum was incubated with 10 μl (50% v/v = 50 μg) of SET1 sepharose. The positions of C5 alpha (110 kD) and C5 beta (70 kD) chains, visible in all 14 samples, are shown by
15   the arrows. Note absence of IgA heavy chain in lane 12. The 50 kD protein that varied in concentration between individuals is IgG Heavy chain and presumably results from natural sero-conversion against SET1. **C.** SET1 binds IgA from human, chimpanzee, baboon, pig, (and possibly rat and horse) and C5 from human, chimpanzee, baboon, sheep, pig, rabbit and goat. SpA-sepharose (lanes
20   A) or SET1-sepharose (= 50μg) (lanes B) was incubated with 10 μl of serum from different species and eluted proteins examined by SDS PAGE under reducing conditions.

   Figure 4. Illustrates that biosensor analysis demonstrates nanomolar binding of SET1 to
25   IgA and C5 through independent sites. Serum IgA was immobilized to a CM5 BIAcore biosensor chip using carbodiimide chemistry. A) Kinetic IgA binding analysis: SET1 was reacted with the immobilized layer at the indicated concentrations at a flow rate of 30μl/min. The data from 20 sensosograms (light grey curves) from 4 independent experiments are shown globally fitted (black
30   solid line), using the BIAevaluation version 2.1 software, to a model describing parallel binding to a heterogeneous ligand model. Two types of sites are

indicated the first $K_D1 = 1.0$ nM, the second $K_D2 = 330$ nM, chi2 = 0.184, n = 4. B) Equilibrium IgA binding analysis: SET1 was reacted with the immobilized layer at the indicated 12 concentrations at a flow rate of 1 μl/min. For each concentration of SET1 the equilibrium binding response to IgA was obtained after 120min. The data shown is one representative experiment. Analysis of 4 independent experiments indicated 2 types of sites; the first $K_D1= 1.1 \pm 0.2$ nM, the second $K_D2= 80 \pm 40$ nM. C) SET1 captured on IgA can simultaneously bind human C5: SET1 (1 μM). was reacted with the immobilized IgA layer (30 μl, flow rate of 10 μl/min) and a subsequent injection was made of either buffer (dashed line) or human C5 (0.3 μM, 30 μl). D) Equilibrium C5 binding analysis: SET1 (1 μM) was reacted with the immobilized IgA layer (30 μl, flow rate of 10 μl/min) and a subsequent injection (122 μl, flow rate of 1 μl/min) was made of the indicated concentrations of human C5 (122 μl). For each concentration of C5 the equilibrium binding response to the SET1:IgA was obtained after 120min. The data shown is one representative experiment fitted to a single binding site. Analysis of 4 independent experiments indicated $K_D= 18 \pm 1$ nM.

Figure 5. Illustrates different alleles of SSL7 bind IgA. A. Amino sequence alignment of known alleles of SET1. Two local clinical isolates (GL1 and GL10) are included in the alignment. Identity (*), strong similarity (:), and weak similarity (.), at each amino acid position is indicated. B. Proteins eluted from immobilized alleles (GL1 and GL10) that differ in 30/201 amino acids (underlined in A) run on SDS PAGE under reducing conditions. Both alleles bound IgA and C5 from two human sera.

Figure 6. Illustrates flow cytometric analysis of IgA binding to granulocytes. IgA-FITC was added at 10 μg to freshly prepared human granulocytes (gray histogram). SET1 (0.1 – 100 μg) (open histograms) was incubated with IgA-FITC prior to

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cell staining (upper panel). SEA was used as a negative control and does not inhibit cell staining by IgA-FITC (lower panel).

Figure 7. Illustrates SET1 inhibits the binding of IgA to rsFcαRI. Biosensor analysis of recombinant SSL7 binding to recombinant CD89 (FcαRI) **A.** No binding of 200 nM SET1 to immobilized FcαRI was detected. **B.** Addition of increasing amounts of SET1 (0-150 nM) progressively prevented the binding of soluble IgA (100nM) to immobilized rsFcαRI. SET1 was incubated with IgA prior to binding the rsFcαRI immobilized chip surface. **C.** The 1min post-injection responses of IgA binding indicate the dose-dependent inhibition by SET1. At stoichiometric addition of SET1 (100nM) to the IgA binding (62 RU) to rsFcαRI was inhibited by 70%.

Figure 8. Illustrates SET1 blocks complement mediated hemolysis of human erythrocytes. **A.** Hemolysis by IB serum of human RBC was inhibited in a dose dependent fashion by recombinant SET1. Maximum inhibition by SET1 was achieved at 0.63 μg for 25μl serum (equivalent to 1.0 nM SET1). **B.** Inhibition of JL serum. Maximum inhibition was achieved at 0.16 μg SET1 equivalent to 0.26 nM). **C.** Addition of recombinant SET3 did not affect hemolysis. Each tube used 25 μl of human serum. Curves represent the average of duplicate.

Figure 9. Illustrates Recombinant SET1 increases bacterial survival in serum. Freshly isolated human serum (500 μl diluted 1:2) was pre-incubated in duplicate with increasing concentrations of either recombinant SET1 or staphylococcal enterotoxin A (SEA) (control) for 30 minutes at 37°C prior to the addition of ~ 10^7 *E. coli* K12 bacteria. Surviving bacteria were counted by plating of appropriate dilutions on agar.

Figure 10. Illustrates the results of single step affinity purification of IgA from human breast milk and bovine colostrums. A representative trace of SSL7 purified IgA

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from human breast milk, bovine or ovine colostrums is shown as absorbance units (AU) at 280nm. 1ml of diluted (40 mg/ml) milk or colostrums was passed through the 0.7ml column at a flow rate of 10 ml/min and bound protein eluted with 50mM glycine pH11.   B.   SDS PAGE of eluted proteins indicates

5   equivalent amounts of bovine secretory component (75 kD) and bovine IgA heavy chain (55 kD) that migrated as a single species ~400 kD under non-reducing conditions that resolved into SC and IgA heavy chain under reducing conditions.

10   **PREFERRED EMBODIMENT(S)**

The following is a description of the present invention, including preferred embodiments thereof, given in general terms. The invention is further elucidated from the disclosure given under the section "Examples" which provides experimental data supporting the invention and specific examples thereof.

15

As mentioned herein before, there has been a recent move to standardise nomenclature used in relation to the SET proteins. Accordingly, they may now be known as SSL (staphylococcal superantigen-like) proteins. In line with this, the proteins SET1 (from strain NCTC6571), SET11 (from strains N315 and Mu50) and SET22 (from strain MW2),

20   each of which may be referred to herein, may now be referred to as alleles of SET1 (SSL7). As described in Lina *et al*, the SSLs are now named in sequential order as they occur on the genome.

The inventors have surprisingly discovered that SET1 proteins bind independently the

25   antibody IgA and the complement factor C5. This has significant application in the purification or isolation of both IgA and C5 from samples. An additional application is in the identification or detection, including quantitation, of IgA and C5 in a sample.

The staphylococcal exotoxin-like proteins (SETs), encoded by genes clustered within the

30   staphylococcal pathogenicity island SaPIn2 are superantigen homologues. The function or role of SETs in unknown but they do not possess any superantigen activity despite

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ancestral relatedness.   The inventors show here that recombinant SET1 expressed in *E. coli* selectively binds to human, chimapanzee, baboon, pig, horse, rat and sheep and cow IgA. They have also shown that SET1 selectively binds serum complement factor C5 from a number of different species tested (including human, goat, sheep, rabbit, chimpanzee,

5   baboon and pig). SET1 blocked binding of IgA to cell surface bound FcαRI (CD89) and stoichiometric addition of SET1 to IgA was sufficient to inhibit binding to purified recombinant FcαR protein. SET1 was also a potent inhibitor of complement-mediated hemolysis. SET1 bound to both monomer and secretory forms of Immunoglobulin A, demonstrating that SET1 and the secretory chain (SC) binding sites on IgA do not overlap.

10   These results indicate that SET1 is a soluble microbial defense factor with multiple activities that targets two important host innate protective mechanisms - Fc receptor mediated phagocytosis and complement mediated inflammatory responses to *S. aureus*.

It should be appreciated that reference to isolation, removal, detection or quantifying the

15   level of IgA and/or C5, may include isolation, removal, detection or quantifying the level of a subunit or monomer of IgA and/or C5, or fragments of such molecules where appropriate.

Further, it should be appreciated that reference to IgA includes reference to isotypes or

20   subclasses of IgA, for example IgA1 and IgA2, which are capable of binding to SET1 or functional equivalents thereof. Similarly, reference to C5 should be taken to include reference to any alternative forms of this molecule which are capable of binding to SET1 or functional equivalents thereof.

25   A first embodiment of the invention relates to a method for isolating IgA and/or C5 from a sample using a SET1 protein or functional equivalent thereof. Generally, the method comprises at least the steps of: bringing SET1 or a functional equivalent thereof in contact with the sample for a period sufficient to allow SET1 or functional equivalent thereof to bind to IgA and/or C5 to form a complex; separating the complex; and, releasing IgA

30   and/or C5 from the complex.

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In a preferred form of this embodiment the method comprises at least the steps of: providing a matrix to which a SET1 protein or functional equivalent is bound; providing a sample; bringing said matrix and said sample into contact for a period sufficient to allow SET1 or functional equivalent thereof to bind to IgA and/or C5 present in the sample; and,

5   releasing IgA and/or C5 from the matrix.

It should be understood that the terms "isolate", or "isolating" and the like indicates that IgA and/or C5 have been separated from at least one contaminating compound. It should be appreciated that 'isolated' does not reflect the extent to which IgA and/or C5 have been

10   purified.

A SET1 protein of the invention may be exemplified by the following amino acid sequence which is provided on GenBank:

SET1   gi|6176432|gb|AAF05587.1|AF188835_1   [Staphylococcus   aureus   subsp.

15   NCTC8325] exotoxin 1 (set1) gene, set1-A allele, complete cds ·

MKLKTLAKATLALGLLTTGVITSEGQAVQAAEKQERVQHLHDIRDLHRYYSSESF

EYSNVSGKVENYNGSNVVRFNPKDQNHQLFLLGKDKEQYKEGLQGQNVFVVQE

LIDPNGRLSTVGGVTKKNNKTSETNTPLFVNKVNGEDLDASIDSFLIQKEEISLKEL

DFKIRQQLVNNYGLYKGTSKYGKIIINLKDENKVEIDLGDKLQFERMGDVLNSKDI

20 ˙ RGISVTINQI [SEQ ID NO:1]

However, there is noted allelic variation in SET1 proteins. The inventors have shown that allelic variation does not alter the binding properties of SET1 to IgA or C5. Accordingly, the invention is applicable to allelic variants of SET1 proteins specifically exemplified

25   herein (for example SET 11 and SET 22 isolated from different strains of *S.aureus*). Such allelic variants generally have at least approximately 80% similarity at the amino acid level. The invention is also applicable to those proteins not considered allelic variants *per se* but which incorporate conservative (as is understood in the art) amino acid substitutions at one or more sites. Additionally, the invention is applicable to proteins or peptides which

30   represent addition/deletion mutants of the SET1 proteins specifically exemplified herein, or those in which non-naturally occurring or modified amino acids have been substituted

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for naturally occurring amino acids at one or more position. The aforementioned variants may be referred to herein as "functional equivalents". In accordance with the invention, "functional equivalents" retain the ability to bind to IgA and C5 from human serum. The term, "bind to" IgA and C5 should not be taken to imply a specific level of binding or

5   affinity between the molecules or that they will have equal affinity for IgA and C5. The binding or affinity is preferably strong enough to allow for one or more of isolation, removal and/or detection of these molecules.

The inventors contemplate the use of SET1 or functional equivalents thereof in the form of

10   fusion proteins, provided the heterologous amino acid sequence does not substantially interfere with binding to IgA and/or C5. Such fusion proteins are also considered to be "functional equivalents" of SET1.

Reference to SET1 proteins and functional equivalents thereof, and the exemplary

15   sequences provided on public databases and otherwise herein, should be taken to include reference to mature SET1 polypeptides excluding any signal or leader peptide sequences or other sequences not present in the mature protein but which may be represented on such databases or in the sequence information contained herein. Persons of general skill in the art to which the invention relates will readily appreciate such mature proteins.

20
Reference to nucleic acids encoding SET1 and its functional equivalents should be taken to include those nucleic acids encompassing the amino acid coding region, and also those which additionally contain regulatory sequences such as promoters, operators or enhancers.

25
Typically, a SET1 protein, or function equivalent thereof, will have an amino acid sequence having at least approximately 80% similarity to that of GenBank AAF05587.1 (SEQ ID NO: 1 above). Preferably, there will be at least approximately 85% amino acid similarity, more preferably at least approximately 90% similarity.

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It should be noted that SET1 proteins and their functional equivalents may be of human or any other animal origin.

Specific examples of further SET1 proteins of use in the invention include:

5

[SEQ ID NO: 2] – provided on GenBank database

>SET1 gi|6176432|gb|AAF05587.1|AF188835_1 Staphylococcus aureus exotoxin 1 (set1) gene, set1-A allele, complete cds

MKLKTLAKATLALGLLTTGVITSEGQAVQAAEKQERVQHLHDIRDLHRYYSSESF
10   EYSNVSGKVENYNGSNVVRFNPKDQNHQLFLLGKDKEQYKEGLQGQNVFVVQE
LIDPNGRLSTVGGVTKKNNKTSETNTPLFVNKVNGEDLDASIDSFLIQKEEISLKEL
DFKIRQQLVNNYGLYKGTSKYGKIIINLKDENKVEIDLGDKLQFERMGDVLNSKDI
RGISVTINQI

15   [SEQ ID NO: 3] – provided on DDBJ database

>SET11_N315 gi|13700317|dbj|BAB41615.1| exotoxin 11 [Staphylococcus aureus subsp. aureus N315]

MKLKTLAKATLALGLLTTGVITSEGQAVHAKEKQERVQHLYDIKDLYRYYSSESF
EFSNISGKVENYNGSNVVRFNQEKQNHQLFLLGKDKDKYKKGLEGQNVFVVKEL
20   IDPNGRLSTVGGVTKKNNKSSETNTHLFVNKVYGGNLDASIDSFLINKEEVSLKEL
DFKIRKQLVEKYGLYKGTTKYGKITINLKDEKKEVIDLGDKLQFERMGDVLNSKD
IQNIAVTINQI

[SEQ ID NO: 4] – provided on GenBank database
25   >SET11_Mu50 gi|15923416|ref|NP_370950.1| exotoxin 11 [Staphylococcus aureus subsp. aureus Mu50]

MKLKTLAKATLALGLLTTGVITSEGQAVHAKEKQERVQHLYDIKDLYRYYSSESF
EFSNISGKVENYNGSNVVRFNQEKQNHQLFLLGKDKDKYKKGLEGQNVFVVKEL
IDPNGRLSTVGGVTKKNNKSSETNTHLFVNKVYGGNLDASIDSFLINKEEVSLKEL
30   DFKIRKQLVEKYGLYKGTTKYGKITINLKDEKKEVIDLGDKLQFERMGDVLNSKD
IQNIAVTINQI

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[SEQ ID NO: 5] – provided on GenBank database

>SET22_MW2   gi|21282117|ref|NP_645205.1| ORFID:MW0388~exotoxin   homolog

[Genomic island nu Sa alpha2] [Staphylococcus aureus subsp. aureus MW2]

5   MKLKTLAKATLALGLLTTGVITSEGQAVQAKEKQERVQHLYDIKDLHRYYSSESF

EFSNISGKVENYNGSNVVRFNQENQNHQLFLSGKDKDKYKEGLEGQNVFVVKELI

DPNGRLSTVGGVTKKNNQSSETNTPLFIKKVYGGNLDASIESFLINKEEVSLKELDF

KIRQHLVKNYGLYKGTTKYGKITFNLKDGEKQEIDLGDKLQFEHMGDVLNSKDIQ

NIAVTINQI

10

[SEQ ID NO: 6] – novel SET1 allelic variant isolated by inventors

>SET1 GL10 S.aureus Greenlane

KEKQERVQHLYDIKDLHRYYSSESFDFSNISGKVENYNGSNVVRFNQDGQNHQLF

LLGEDKAKYKQGLEGQNVFVVKELIDPNGRLSTVGGVTKKNNQSSETNTPLFVKK

15   VYGGNLDASIESFSINKEEVSLKELDFKIRQHLVKNYGLYKGTTKYGKITFNLKDG

EKKEIDLGDKLQFEHMGDVLNSKDIQNIAVTLKQI

[SEQ ID NO: 7] – novel SET1 allelic variant isolated by inventors

>SET1 GL1 S.aureus Greenlane

20   KEKQERVQHLYDIKDLHRYYSSESFEFSNISGKVENYNGSNVVRFNQEKQNHQLF

LLGEDKAKYKQGLQGQDVFVVKELIDPNGRLSTVGGVTKKNNQSSETNIHLLVN

KLDGGNLDATNDSFLINKEEVSLKELDFKIRKQLVEKYGLYQGTSKYGKITIILNG

GKKQEIDLGDKLQFERMGDVLNSKDINKIEVTLKQI

25   Nucleic acids encoding SET1 proteins and functional equivalents of the invention will be

appreciated having regard to the information herein and the known degeneracy in the

genetic code. However, exemplary nucleic acids include:

[SEQ ID NO: 8] – provided on GenBank database

30   >set1|AF188835|[Staphylococcus aureus subsp. aureus NCTC6571]

```
     1 atgaaattaa aaacgttagc taaagcaaca ttagcattag gtttattaac tactggtgtc
    61 attacatcag aaggtcaagc agttcaagcg |gcagaaaaac aagagagagt acaacattta
```

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```
121  catgatatta  gagatttaca  tcgatactac  tcatcagaaa  gtttcgaata  tagtaatgtt
181  agtggtaagg  ttgaaaacta  caatggttct  aacgttgtac  gctttaaccc  aaaagatcaa
241  aatcaccaat  tattcttatt  aggaaaagat  aaagaacaat  ataaagaagg  tctacaaggc
301  caaaatgtct  ttgtagtaca  agaattaatt  gatccaaacg  gcagactatc  tactgttggt
361  ggtgtaacga  agaaaaacaa  caaaacttct  gaaactaata  cacctttatt  tgttaataaa
421  gttaatggtg  aagatttaga  tgcatcaatt  gactcatttt  taatccaaaa  agaagaaatc
481  tcattaaaag  agcttgattt  caaaattaga  caacaattag  ttaataatta  cggattatat
541  aaaggtacat  ctaaatacgg  taaaatcatt  atcaatttga  aagacgaaaa  taaagtagaa
601  attgatttag  gtgataaatt  acaattcgag  cgcatgggcg  atgtgttgaa  tagtaaagac
661  attagaggta  tatcagtcac  tattaaccaa  atttaa
```

[SEQ ID NO: 9] – provided on GenBank database

>exotoxin 11 | BAB41615.1 | [Staphylococcus aureus subsp. aureus N315].

```
  1  atgaaattaa  aaacgttagc  taaagcaaca  ttggcattag  gcttattaac  tactggtgtg
 61  attacatcag  aaggccaagc  agtccacgca  aaagaaaagc  aagagagagt  acaacattta
121  tatgatatta  aagacttata  tcgatactac  tcatcagaaa  gttttgaatt  cagtaatatt
181  agtggtaagg  ttgaaaacta  taacggttct  aacgttgtac  gctttaacca  agaaaaacaa
241  aatcaccaat  tattcttatt  aggaaaagat  aaagataaat  ataaaaaagg  ccttgaaggc
301  cagaatgtct  ttgtggtaaa  agaattaatt  gatccaaacg  gtagactatc  tactgttggt
361  ggtgtgacta  agaaaaataa  caaatcttct  gaaactaata  cacatttatt  tgttaataaa
421  gtgtatggcg  gaaatttaga  tgcatcaatt  gactcatttt  taattaataa  agaagaagtt
481  tcactgaaag  aacttgattt  caaaattaga  aagcaattag  ttgaaaaata  tggtttatat
541  aaaggtacga  ctaaatacgg  taagatcact  atcaatttga  aagacgagaa  aaaggaagta
601  attgatttag  gtgataaact  gcaattcgag  cgcatgggtg  atgtgttgaa  tagtaaggat
661  attcaaaata  tagcagtgac  tattaatcaa  atttaa
```

[SEQ ID NO: 10] – provided on GenBank database

>exotoxin 11 | NP_370950.1 | [Staphylococcus aureus subsp. aureus Mu50].

```
  1  atgaaattaa  aaacgttagc  taaagcaaca  ttggcattag  gcttattaac  tactggtgtg
 61  attacatcag  aaggccaagc  agtccacgca  aaagaaaagc  aagagagagt  acaacattta
121  tatgatatta  aagacttata  tcgatactac  tcatcagaaa  gttttgaatt  cagtaatatt
181  agtggtaagg  ttgaaaacta  taacggttct  aacgttgtac  gctttaacca  agaaaaacaa
241  aatcaccaat  tattcttatt  aggaaaagat  aaagataaat  ataaaaaagg  ccttgaaggc
301  cagaatgtct  ttgtggtaaa  agaattaatt  gatccaaacg  gtagactatc  tactgttggt
361  ggtgtgacta  agaaaaataa  caaatcttct  gaaactaata  cacatttatt  tgttaataaa
421  gtgtatggcg  gaaatttaga  tgcatcaatt  gactcatttt  taattaataa  agaagaagtt
481  tcactgaaag  aacttgattt  caaaattaga  aagcaattag  ttgaaaaata  tggtttatat
541  aaaggtacga  ctaaatacgg  taagatcact  atcaatttga  aagacgagaa  aaaggaagta
601  attgatttag  gtgataaact  gcaattcgag  cgcatgggtg  atgtgttgaa  tagtaaggat
661  attcaaaata  tagcagtgac  tattaatcaa  atttaa
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[SEQ ID NO: 11] – provided on GenBank database

>exotoxin 22 | NC_003923.1 | [Staphylococcus aureus subsp. aureus MW2]

```
  1 gtgaaattaa aaacgttagc taaagcaaca ttggcattag gcttattaac tactggtgtg
 61 attacatcag aaggccaagc agtgcaagca aaagaaaagc aagagagagt acaacatttta
121 tatgatatta aagacttaca tcgatactac tcatcagaaa gttttgaatt cagtaatatt
181 agtggtaagg ttgaaaatta taacggttct aacgttgtac gctttaacca agaaaatcaa
241 aatcaccaat tattcttatc aggaaaagat aaagataaat ataagaagg ccttgaaggc
301 cagaatgtct ttgtggtaaa agaattaatt gatccaaacg gtagactatc tactgttggt
361 ggtgtaacga agaaaaataa ccaatcttct gaaactaata cacctttatt tataaaaaaa
421 gtgtatggcg gaaatttaga tgcatcaatt gaatcatttt taattaataa agaagaagtt
481 tcactgaaag aacttgattt caaaattaga caacatttag ttaaaaatta tggtttatat
541 aaaggtacga ctaaatacgg taagatcact ttcaatttga aagatggaga aaagcaagaa
601 attgatttag gtgataaatt gcaattcgag cacatgggcg atgtgttgaa tagtaaggat
661 attcaaaata tagcagtgac tattaatcaa atttaa
```

[SEQ ID NO: 12] – nucleic acid encoding novel SET1 protein [SEQ ID NO: 6]

>set1 | GL10 isolate

```
  1 aaagaaaaac aggaacgtgt tcagcacctg tacgacatca aagacctgca
 51 ccgttactac tcctccgaat ccttcgaatt ctccaacatc tccggtaaag
101 ttgaaaacta caacggttcc aacgttgttc gtttcaacca ggaaaaacag
151 aaccaccagc tgttcctgct gggtgaagac aaagctaaat acaaacaggg
201 tctgcagggt caggacgttt tcgttgttaa agaactgatc gacccgaacg
251 gtcgtctgtc caccgttggt ggtgttacca aaaaaaacaa ccagtcctcc
301 gaaaccaaca tccacctgct ggttaacaaa ctggacggtg gtaacctgga
351 cgctaccaac gactccttcc tgatcaacaa agaagaagtt tccctgaaag
401 aactggactt caaaatccgt aaacagctgg ttgaaaaata cggtctgtac
451 cagggtacct ccaaatacgg taaaatcacc atcatcctga cggtggtaa
501 aaaacaggaa atcgacctgg gtgacaaact gcagttcgaa cgtatgggtg
551 acgttctgaa ctccaaagac atcaacaaaa tcgaagttac cctgaaacag
601 atctaa
```

[SEQ ID NO: 13] – nucleic acid encoding novel SET1 protein [SEQ ID NO: 7]

>set1 | GL1 isolate

```
  1 aaagaaaaac aggaacgtgt tcagcacctg tacgacatca aagacctgca
 51 ccgttactac tcctccgaat ccttcgaatt ctccaacatc tccggtaaag
101 ttgaaaacta caacggttcc aacgttgttc gtttcaacca ggaaaaacag
151 aaccaccagc tgttcctgct gggtgaagac aaagctaaat acaaacaggg
201 tctgcagggt caggacgttt tcgttgttaa agaactgatc gacccgaacg
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- 20 -

```
251   gtcgtctgtc caccgttggt ggtgttacca aaaaaaacaa ccagtcctcc
301   gaaaccaaca tccacctgct ggttaacaaa ctggacggtg gtaacctgga
351   cgctaccaac gactccttcc tgatcaacaa agaagaagtt tccctgaaag
401   aactggactt caaaatccgt aaacagctgg ttgaaaaata cggtctgtac
451   cagggtacct ccaaatacgg taaaatcacc atcatcctga acggtggtaa
501   aaaacaggaa atcgacctgg gtgacaaact gcagttcgaa cgtatgggtg
551   acgttctgaa ctccaaagac atcaacaaaa tcgaagttac cctgaaacag
601   atctaa
```

As noted above, the inventors have identified two novel SET1 proteins represented by the amino acid sequences SEQ ID Nos: 6 and 7, and representative nucleic acids encoding them (SEQ ID Nos: 12 and 13). Accordingly, the invention relates to isolated forms of these proteins and nucleic acids encoding them. In this context an "isolated" SET 1 protein or nucleic acid is one which has been identified and separated from at least one contaminant compound (for example a nucleic acid) with which it is associated in its natural state. Accordingly, it will be understood that isolated proteins and nucleic acids are in a form which differs from the form or setting in which they are found in nature. It should be appreciated that 'isolated' does not reflect the extent to which the protein or nucleic acid molecule has been purified.

It should be appreciated that the invention extends to novel SET1 encoding nucleic acids as defined herein (SEQ ID Nos: 12 and 13) which are in association with heterologous nucleic acids, such as regulatory regions and nucleic acids encoding heterologous fusion peptides. The invention also extends to nucleic acid vectors or constructs containing SET1 encoding nucleic acids as defined herein. Such constructs may find use in cloning and expression. The invention also relates to fusion proteins containing the novel SET1 proteins described herein (SEQ ID Nos: 12 and 13).

A SET1 protein or functional equivalent thereof may be made by any number of standard techniques known in the art, having regard to the amino acid and nucleic acid sequences identified herein before. By way of example, they may be isolated from natural sources such as purification from crude culture supernatants of strains of *Staphylococcus aureus* known to produce SET1, produced recombinantly having regard to the genetic code, or

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produced by chemical protein synthesis An exemplary protocol for obtaining recombinant SET1 is described herein after under the heading "Examples".

In accordance with a preferred form of the invention, IgA and/or C5 are captured or
5   isolated using affinity chromatography however a skilled person may readily recognise alternative techniques.   Generally, an affinity column is prepared combining a SET1 protein or functional equivalent thereof, suitably immobilised on a support resin or matrix.

Any appropriate support resin as known in the art may be used. As it will be appreciated,
10   choice of support resin may depend on the means by which SET1 or a functional equivalent thereof is to be immobilised on it. Preferable support resins include Sepharose such as Sepharose 4B, cyanogen bromide-activated (CNBr-activated) Sepharose, AH-Sepharose 4B and CH-Sepharose 4B, activated CH-Sepharose 4B, Epoxy-activated Sepharose 6B, activated Thiol-Sepharose 4B, Thiopropyl-Sepharose 6B, covalently cross-
15   linked Sepharose (sepharose Cl), and other resins such as nickel chelate resins, cellulose, polyacrylamide, dextran. Such resins may be purchased for example from Pharmacia Biotech. However, a skilled person may produce a resin themselves using methodology standard in the art

20   While the inventors have found that it is not necessary to use spacer molecules it should be appreciated that where desirable, and where one is not present on a resin as it may be purchased or manufactured, a spacer molecule may be added to the resin. Such spacer molecule may, in certain circumstances, facilitate the attachment of the ligand (SET1 or a functional equivalent thereof) to the resin, and also facilitate efficient chromatographic
25   isolation of IgA and/or C5. Relevant spacer molecules will readily be appreciated by persons of ordinary skill in the art.

In addition, cross-linking of a support resin, or activation of resins may help facilitate chromatographic separation. Accordingly the invention encompasses this. While support
30   resins which have been cross-linked and/or activated may be readily purchased (for

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example, Sepharose Cl or CNBr-acitivated Sepharose) skilled persons will readily appreciate methods for achieving such results themselves

5   It will be appreciated that SET1 proteins or their functional equivalents may be chemically modified where necessary and to facilitate attachment to the support resin while not destroying its ability to bind C5 and/or IgA.

Once the support resin is prepared and any modifications made to it and/or a SET1 protein or functional equivalent, the SET1 protein or functional equivalent may be immobilized on
10   the support resin using standard methodology. By way of example, the protein and the resin may simply be mixed for a period of time (by way of example, 2 hours) to allow for attachment of the protein to the resin. Subsequently, any active groups which may remain on the resin may be blocked by mixing with a buffer such as Tris at pH 8.0 for a period of time (for example 2 hours). The protein-resin may then be washed in an appropriate
15   buffer, such as PBS, then suspended in an appropriate buffer and stored. In a preferred from of the invention where a Sepharose resin is used, the protein-resin is stored 1:1 in a PBS/0.025% NaN$_3$ buffer at 4°C until desired to be used. Further preferred methodology is exemplified herein after under the heading "Examples".

20   SET1 or a functional equivalent thereof may be combined with the support resin in any desired ratio. In a preferred form of the invention using CNBr-activated Sepharose 4B, SET1 is combined at 7mg of protein/ml of wet gel Sepharose. This typically results in a concentration of approximately 5mg protein/ml of Sepharose gel.

25   Once the affinity matrix or resin is prepared as mentioned herein before it may be formed into a column according to standard techniques readily known in the field. The column may then be washed with an appropriate buffer to prepare it for taking a sample. Such appropriate buffer includes for example PBS, or any other neutral pH buffer containing isotonic concentrations of NaCl. A sample may then be loaded onto the column and
30   allowed to pass over the column. In this step, IgA and/or C5 present in a sample will adsorb to the column resin or matrix.

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Appropriate "samples" from which IgA and/or C5 may be captured or isolated include milk, colostrums, serum, tears, saliva, secretions from the gut or any other secretions containing either IgA or complement C5. The sample may be of human, or other animal origin. In one preferred embodiment, the sample is cows milk or collostrum. In another preferred embodiment, the sample is human serum.

Once a sample has passed over the column it will generally be washed with an appropriate buffer to remove unbound or non-specific proteins or other compounds which may have been present in the original sample. Skilled persons will readily appreciate an appropriate buffer suitable for use. However, by way of example, a PBS/300mM NaCl buffer may be advantageously used.

IgA may be eluted from the column using 100mM glycine at pH 3.0. In a specific example, 10 column volumes of glycine is used. However, it will be appreciated that this may be varied. The IgA will generally be eluted into any buffer which is adapted to neutralize the elution buffer. The inventors have found that 1M Tris pH 8.0 is appropriate.

C5 may be eluted under similar conditions. The inventors note that C5 elutes at slightly higher pH. Accordingly, a low pH buffer such as 50mm acetate pH 3.5 may preferably be used for C5.

Following elution or release of IgA and/or C5 from the matrix or column, IgA and/or C5 may be further purified via any number of standard techniques. For example, eluates may be dialysed, or run through an affinity column of the invention again.

It should be appreciated that a chromatographic column in accordance with the invention may be gravity fed, or fed using positive or negative pressure. For example, FPLC and HPLC are applicable to a method of the invention.

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Skilled persons will readily appreciate how to implement an HPLC system in relation to the present invention having regard to the information herein and standard methodology documented in the art.

5   Persons of ordinary skill in the art to which the invention relates will readily appreciate how scale up of bench top columns may be achieved. For example, one may increase volume of the affinity column consistent with the volume of sample to be processed. Commercial scale may be dependent on ensuring that the amount of coupled SET1 saturates the amount of ligand to be bound. Alternatively, large amounts of sample may be
10   processed by repeated processing through a smaller SET1 affinity column. This has the advantage of not requiring so much SET1 but does rely on the reusability of the SET1 for recycling. The inventors have found that SET1 is very stable when used for purification of IgA and/or C5 and can be reused many times without loss of binding activity.

15   It should be appreciated that an affinity matrix can also be used in batch wise fashion where the solid matrix is added directly to the sample rather than passing the sample through a column. This offers simplicity, but may result in a less clean sample. Such techniques require a step to separate the matrix from the solution or sample. This is normally achieved by gravity sedimentation and decantation of the supernatant followed
20   by washing, or separation of the affinity matrix by low pressure gravity or suction filtration.

The present invention has the advantage of providing a one step system for isolating IgA and/or C5. It should be appreciated that there may be instances where it is desirable to
25   obtain a biological sample that is free from IgA and/or C5. The present invention will allow for substantial removal of these molecules from a sample. The techniques described hereinbefore are suitable for achieving this end. It should be appreciated that where removal of IgA and/or C5 is the objective (as opposed to capture and purification of IgA and/or C5) it would not be necessary to release IgA and/or C5 from any SET1 (or
30   functional equivalent) to which it is bound.

- 25 -

In addition, the inventors demonstration of binding between SET1 and IgA and/or C5, provides a means for detecting the presence, and quantifying the level, of these proteins in a sample. This has diagnostic significance in determining sero-conversion of mucosal immunity (IgA), complement competency (C5) and in detecting abnormalities in these

5   proteins (for example deficiencies, or increased expression, in a subject). Diagnostic methods involving detection and/or quantitation of IgA and/or C5 may find particular use in assessing the immune competence of an individual. For example, reduced salivary IgA levels are believed to be linked to increased susceptibility to infection. This may be of particular benefit to assessing athletes during intensive training regimes, and also assessing

10   the immune status of invalids. Knowledge of immune competence may allow for more informed and individualised approaches to training schedules, nutrition, and medication regimes.

In accordance with the above, the invention also provides methods for detecting the

15   presence, and/or quantifying the level of IgA and/or C5 in a sample. The method will generally comprise the steps: contacting a sample with SET1 or functional equivalent thereof for a period sufficient to allow SET1 or functional equivalent thereof to bind to IgA and/or C5; and, detecting bound SET1 or functional equivalent thereof. The method preferably includes the further step of determining the level of bound SET1 or a functional

20   equivalent thereof. Such a method is applicable to any sample which may contain IgA and/or C5. It is applicable to samples from humans and other animals.

Persons of skill in the art to which the invention relates will appreciate means by which these proteins can be detected and/or quantified. However, by way of example SET1 or a

25   functional equivalent thereof may first be conjugated to peroxidase or alkaline phosphatase by chemical cross-linking using standard methods to produce a staining reagent. Samples can be added to ELISA plates and SET1 or functional equivalent thereof can be added at a fixed concentration to bind to any IgA or C5 bound to the plastic plate surface. Following washing, the amount of SET1 or functional equivalent thereof can be quantified by

30   measuring the amount of peroxidase or alkaline phosphatase bound using established colorimetric methods that result in the production of a coloured compound which can be

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measured in an ELISA plate reader. The levels of IgA/C5 in the sample can be determined by comparing results against a standard curve of a known sample of either IgA or C5. An alternative example is to utilise a sandwich ELISA employing an anti-SET1 specific antibody. In this case the anti-SET1 antibody is conjugated to either peroxidase or alkaline 5   phosphatase. After the SET1 or functional equivalent thereof has incubated with the sample on the ELISA plate and excess washed away, the anti-SET1 antibody linked to the enzyme is incubated and washed clean.

EXAMPLES

10   **Methods**

SET1 protein expression and purification

The *SET1* gene was amplified by PCR from the genomic DNA of two local clinical isolates of *Staphylococcus aureus* (designated GL1 and GL10) obtained from Greenlane Hospital (Auckland, New   Zealand)   using   the   primer   sequences   (Forward-BamH1)   (Reverse-EcoR1) 15   CGGGATCCAAAGAAAAGCAAGAGAGAG   and GGAATTCTTAAATTTGTTTCAAAGTCAC. The PCR fragment was sub-cloned into the expression vector pET32a-3C and expressed in *E. coli* (AD494(DE3)pLysS) as an N-terminal thioredoxin fusion protein. The fusion protein was purified by Ni^{2+} affinity chromatography, cleaved using 3C protease, and subjected to Ni^{2+} affinity chromatography again to separate SET1 20   from thioredoxin as previously described (11). Purified SET1 was passed through a polymyxin B-agarose column to remove residual *E. coli* endotoxins (Sigma).

Serum and secreted samples

Primate sera were provided by kind donation from Auckland Zoo. Fresh sera from rabbit, 25   cow, mice, pigs, horse, sheep and rat was obtained from the Auckland University Animal Research Unit. Human breast milk was provided as a kind donation from a lactating female laboratory technician. Colostrums from cow and sheep were provided by Dr Colin Prosser (AgResearch, New Zealand). Saliva and tears were collected from laboratory individuals and stored under sterile conditions at 4°C.

30

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Bacterial Strains

Staphylococcal strains GL1 and GL10 are part of a large collection of Staphylococcal strains isolated from patients with endocarditis or bacteremia from Green Lane Hospital, Auckland, New Zealand.

5

Production of recombinant FcαRI protein.

Soluble FcαRI protein was produced by recombinant baculovirus infection of SF21 insect cells and purified by nickel affinity chromatography as previously described (26).

10 *Coupling of proteins to sepharose*

Protein in PBS, pH 8.0 was added to CNBr-activated Sepharose 4B (Pharmacia Biotech) at 5-7mg protein/ml of wet gel sepharose and mixed by rotation at room temperature for 2 hrs. Remaining active groups were blocked with the addition of Tris pH 8.0 to 100mM and incubating for 2 hrs. The protein sepharose was washed a total of six times in PBS

15 then suspended 1:1 in PBS/0.025% NaN$_3$ and stored at 4°C. Coupling typically resulted in concentrations of approximately 5 mg protein/ml of sepharose gel. Coupled gel was stored in PBS, 0.1% azide at 50% v/v at 4°C.

SET1 binding assays

20 10μl of SET1-sepharose (50% v/v, equivalent to approximately 50μl of SET-1) or protein A-sepharose (50% v/v) suspension was added to 10μl serum or 100μl secretion respectively and the volume made up to 500μl with lysis buffer (1% TX-100, 1% bovine hemoglobin, 140mM NaCl, 10mM Tris.Cl pH 8.0, 0.025% NaN$_3$, 1mM PMSF, 1mM iodoacetamide) and incubated with rotation for 1 hour at 4°C. Sepharose without any

25 protein coupled was used as a negative control to determine proteins bound independently of SET1. The samples were washed three times using wash buffer (1% TX-100, 0.1% SDS, 1% deoxycolate, 500mM NaCl, 10mM Tris.Cl pH 8.0), once with TSA (10mM Tris.Cl pH 8.0, 140mM NaCl, 0.025% NaN$_3$), and once with 50mM Tris pH 6.8. Proteins were solubilised by boiling for 2-5 min in 10μl of SDS-PAGE sample buffer prior to

30 running on a 10 or 12.5% SDS-PAGE gel.

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Western Blot Analysis

Proteins bound by SET1-sepharose or protein A-sepharose were resolved on either 10% or 12.5% SDS-PAGE gels under reducing conditions then transferred to a nitrocellulose membrane using a BioRad transblot apparatus (BioRad Laboratories). The nitrocellulose

5   membrane was incubated for 1 hour at room temperature in TTBS (140 mM NaCl, 10mM Tris.Cl pH 7.6, 0.1% Tween 20) containing 5% (w/v) nonfat milk powder to block non-specific binding. Goat anti-IgA, anti-IgD, anti-IgG, or anti-IgM (Kallestad) was incubated at 1 in 5000 dilution with the membrane fragments for 1 hour at room temperature in TTBS. After 3 x 15 minute washes in 5 ml TTBS the membrane was incubated with a 1 in

10   4000 dilution (in TTBS) of biotin labeled rabbit anti-goat IgG (Sigma) for 1 hour at room temperature followed by 3 x 5ml washes in TTBS. The membranes were then incubated with a 1 in 4000 dilution of peroxidase labeled avidin (Dako), washed 3 times in TTBS, and analyzed using an ECL Western blotting detection kit (Amersham Pharmacia Biotech) as per the manufacturer instructions.

15

Flow cytometric analysis

Polyclonal human IgA was purified from normal human serum by SET1-sepharose affinity chromatography). 1 mg of IgA was incubated with 1 mg fluorescein isothiocyante (FITC Sigma) for 1 hour at 4°C then separated from free FITC by chromatography on a 5.0ml column of

20   Sephadex G25 (Pharmacia). Leukocytes were prepared from whole human blood by first diluting 10-fold with 0.85% ammonium chloride solution to lyse erythrocytes and centrifuging remaining white cells at 2500 rpm x 10 min. Leukocytes were suspended at $1x10^7$ cells/ml in PBS/2% FCS. A 2-fold dilution series from 100μg to 6.25μg of SET1 or SEA was prepared in 100μl PBS/2% FCS. 10μg polyclonal IgA-FITC was added to each dilution of SET1 and incubated for 15 min at

25   RT in the dark. 50μl of freshly prepared leukocytes ($5x10^5$ cells) was added and incubated for 15 min at RT in the dark. Cells were then fixed with the addition of 300μl 8% formaldehyde and incubating for 3 min at RT in the dark. Cells were washed twice with 1ml PBS/2% FCS, centrifuged at 400 x g for 1min, and resuspended in 0.5ml PBS/2% FCS. The cells were analyzed by flow cytometry (FACS Analyser, Becton and Dickinson) with selective gating on the

30   Granulocyte population.

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HPLC affinity purification of secretory IgA from human breast milk, bovine and ovine colostrums

An HPLC affinity column was generated by coupling 5 mg of purified recombinant SSL7 to 0.7gm of POROS CNBr activated media (Perceptive Systems, Cambridge MA)
5   overnight at room temperature in 50mM PO₄ pH8. The 50mm x 5cm HPLC column was mounted on a Biocad HPLC (Perceptive systems). Aliquots of human breast milk or bovine or ovine colostrums diluted to 40 mg/ml and filtered were loaded at 10 ml/min in 50 mM PO₄ pH6.8. Specifically bound protein was eluted with 2 column volumes of 50 mM glycine pH11.0. Collected peaks were analyzed by SDS PAGE. Bands were excised
10 and subjected to peptide mass fingerprinting.

Biosensor analysis

SET1 binding to IgA: Purified human serum IgA (Calbiochem) was immobilized on a CM5 carboxyldextran chip using carbodiimide chemistry to a level of ~300 response units
15 (RU) as described previously (26) using a BIAcore2000™ (BIAcore). Purified SET1 in the concentration range 10 to 200 mM was injected over the chip at a flow rate of 30µl/min. The binding and dissociation kinetics were globally fitted using the BIAevaluation version 2.1 software. The analysis of the equilibrium binding used 120 min injections of SET1 in the concentration range 0.25 to 400 nM SET1. The equilibrium
20 binding response (R$_{eq}$) at 120 min was fitted to the two-site binding model;

$$R_{eq} = B_1 . A/(K_{D1}+A) + B_2 . A/(K_{D2}+A)$$

Where B₁, B₂, K$_{D1}$, K$_{D2}$ are the respective binding capacities and dissociation constants of the two sites and A is the free analyte concentration.

25 Equilibrium C5 binding analysis: SET1 (1µM) was reacted with the immobilized IgA layer (30µl, flow rate of 10µl/min) and a subsequent injection (122µl, flow rate of 1µl/min) was made of human C5 (Sigma) in the concentration range 2 to 130 nM. For each concentration of C5 the equilibrium binding response to the SET1:IgA was obtained after 120min as the response above that of the injection of buffer alone. The data was fitted to
30 the single binding site model;

$$R_{eq} = B . A/(K_D+[A]).$$

- 30 -

The biosensor analysis of SET1 inhibition of IgA binding to FcαRI used rsFcαRI produced by recombinant baculovirus infection of SF21 insect cells and purified by nickel affinity chromatography (26). Briefly rsFcαRI was immobilized on a CM5 carboxyldextran chip for high affinity 2:1 receptor capture of serum IgA described by Wines et al, 2001 (26). Serum IgA (Calbiochem) at 100nM was injected (20µl, 10µl/min) as the analyte and subtraction from a chemically coupled flow cell used to correct for bulk refractive effects. The inhibition of IgA binding was observed by incubating 100nM IgA with 0–150 nM rSET-1 for 30min at 25°C, prior to injection on the BIAcore. A binding response report point 1min after the injection was used to analyze rSET1 inhibition.

Complement mediated hemolytic assay

The assay to detect hemolysis via the alternative complement pathway mediated was followed according to protocols in Handbook of Immunology (27). Serum from normal human individuals was tested for spontaneous hemolytic activity towards allogeneic human red blood cells. Two sera (IB and JL) displayed spontaneous lysis via the alternative activation pathway. Activity was destroyed by heat inactivation (56°C for 30 minutes) or addition of ethylene diamine tetra acetic acid (EDTA) but not ethylene glycol tetra-acetic acid (EGTA) indicating that lysis was via the alternative pathway. Fresh human erythrocytes were prepared by repeated washing in Gelatin/Veronal buffered saline (GVB = 50mM diethyl barbiturate pH7.4, 1% gelatin, 0.15M NaCl). GVB++ buffer is GVB with $CaCl_2$ and $MgCl_2$ added to 0.06 mM and 0.4 mM respectively. GVBE buffer was GVB with 0.4 mM $MgCl_2$ and 10 mM EGTA. Erythrocytes were standardized at 2 x 10^8 cells/ml in ice cold GVB++. Immediately prior to use, 50 µl of serum diluted 1:2 with GVB++ buffer (25 µl equivalent), was incubated for two hours at 37°C with varying concentrations of SET1 protein in duplicate in 12 x 75 mm borosilicate glass tubes. 100 µl human erythrocytes and 50 µl of GVB++ buffer were added and the tubes incubated for 1 hour at 37°C. Total hemolysis was measured by adding 100 µl of water instead of GVB++. To provide a negative control, GVB++ buffer replaced the serum. After 1 hour, 1.2 ml of ice cold 0.15M NaCl was added, tubes were centrifuged at 1250 rpm and hemolysis was measured by the absorbance at 412nm of the supernatant.

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Serum bactericidal assay

500 µl aliquots of 1:2 diluted fresh human serum diluted with Hanks balanced salt solution

(HBSS) were pre-incubated in duplicate for 30 minutes at 37°C with varying

5   concentrations of recombinant SSL7 or Staphylococcal Enterotoxin A (SEA) diluted with

HBSS in 5 ml borosilicate glass tubes. 10 µl (~10^7 cfu) of a fresh mid-log phase (OD$_{620nm}$

0.15) culture of *E. coli* K12 (DH5α strain) was washed in HBSS and added to each sample

then incubated without mixing for 90 minutes at 37°C. Tubes were placed on ice then 50

µl of appropriate dilutions (up to 1,000,000 fold) were plated onto LB plates and cultured

10   for 24hrs at 37°C. Surviving colonies were counted the following day.

Peptide mass fingerprinting and protein identification

Polypeptides were excised from coomassie blue stained SDS PAGE gel, cut into small

cubes and subjected to overnight digestion with trypsin (Promega protein sequencing grade

15   500 IU/mg; Promega, Madison, USA, V511A 11652007) as described previously with

minor modifications (29). Digests were dried down in a vacuum dessicator then

resuspended in 50µl of 0.1% acetic acid /0.005% heptafluorobutyric acid in high purity

water for injection into an Agilent 1100 series ion-trap mass spectrometer. Peptide mass

profiles were analyzed on the MASCOT database (www.matrixscience.com) for protein

20   identification.

Results

SET1 binds IgA and complement C5 from human serum.

Recombinant SET1 (Fig. 1, lane 3), isolated from the GL1 strain, and recombinant soluble

25   FcαRI CD89 (Fig. 1, lane 2) were produced to a high level of purity from *E. coli* and

baculovirus infected Sf21 cells respectively (26). Recombinant SET1 was incubated with

normal human peripheral blood lymphocytes to test for superantigen activity using a

standard 3-day PBL proliferation assay. No proliferative activity was observed over the

entire range of SET1 concentrations used (0.1pg/ml − 10 µg/ml) confirming lack of

30   superantigen activity (data not shown). Binding to a soluble form of HLA-DR1 was

undetectable by BIAcore analysis (data not shown).

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Recombinant SET1 was chemically coupled to sepharose to investigate serum proteins that might bind SET1 directly. Four serum proteins were routinely purified by SET1 from all human sera tested (except for one individual – see below). The two predominant

5   polypeptides of ~60 kD and ~27 kD were observed under reducing conditions but under non-reducing SDS PAGE migrated as a single high MW protein consistent with immunoglobulin heavy and light chain polypeptides (fig. 2a, lane 3). The identity of the polypeptides excised from the SDS PAGE gel was confirmed by MALDI-TOF mass spectrum fingerprinting (performed by the Australia Proteome Analysis Facility). The

10 60kD band was unequivocally identified as human IgA heavy chain while the 27kD band was confirmed as the Ig light chain. In addition to IgA H and L-chains, two additional polypeptides with molecular weights of 110 kD and 75 kD were also purified by SET1. Although these were less predominant than IgA, their relative abundance to IgA was the same in all individuals tested (except one). Unambiguous identification of these

15 polypeptides was again made by MALDI-TOF mass spectrometry (Australian Proteome Analysis Facility) from bands excised from the SDS PAGE. All samples gave unequivocal matches to human complement component C5 which in serum is composed of two covalently linked chains; C5α chain (115kD) and C5β chain (75kD) consistent with the sizes of the bands observed on SDS-PAGE.

20
Proteomic analysis identified IgA as the predominant species bound by SET1 in serum, but could not exclude other isotypes that might be present in lower abundance. SET1 purified Ig H-chain was compared by SDS PAGE to proteins purified by staphylococcal protein A sepharose (SpA-sepharose) that predominantly binds the IgG isotype but also weakly binds

25 other isotypes in humans. The SpA purified IgG H-chain (~50kD) (fig. 2B lane 1) was sufficiently separated in size by SDS PAGE from IgA H-chain (~60kD) (fig. 2B lane 2) to show that very little IgG isotype was bound by SET1. To further confirm this, the SET1 purified proteins were reacted with isotype specific anti-sera by Western blot. Only the anti-IgA antiserum reacted with the protein purified by SET1. Anti-IgG, IgD and IgM

30 were negative (Fig. 2C lane 2). In contrast, all antisera reacted with the SpA purified protein (Fig. 2C lane 1), but the anti-IgG sera was strongest consistent with cross-reactivity

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of each of the isotype specific anti-sera towards IgG heavy chain. This confirmed that SET1 was selective for the IgA isotype.

SET1 binds both serum and secreted IgA from different species.

5   Human secretions were tested for secretory IgA binding to SSL7. In these experiments, 10µl of serum and 100µl human breast milk, saliva and tears were reacted with 10 µl 50% (v/v) SSL7 sepharose (an equivalent of ~50 µg of coupled SSL7). SSL7 bound IgA from all samples (Figure 3A). Monomeric IgA along with the C5 α and β-chain polypeptides were bound from the serum sample, while secretory IgA with the additional 75 kD

10   secretory component (SC) were observed in the breast milk, saliva and tear samples. The SC from human breast milk was noticeably smaller than SC from saliva or tears. Nevertheless its identity was confirmed by peptide mass fingerprinting and so the size difference was presumed to be a result of either an alternative proteolytic cleavage of the poly Ig receptor in mammary tissue and/or variable glycosylation differences.

15

SET1 binding of serum IgA might conceivably be a result of a profound sero-conversion through prior immunization with SET1 producing *S. aureus*. If this were true, then the amount of IgA purified by SET1 from the same volume of sera would be expected to vary between individuals. 10 µl aliquots of serum from 14 individuals were incubated with an

20   equivalent, saturating amount of SET1 sepharose and examined by SDS PAGE. The amount of IgA bound by 13/14 normal volunteers was uniform and consistent suggesting that binding was not unlikely to be via idiotypic regions of the IgA protein. Further studies confirmed that a saturating amount of SET1 extracted all the IgA present in serum with little IgA remaining following a single pass over SET1 sepharose (data not shown). Some

25   individuals (lane 2, 10, 11 and 14 for example) had an additional band at 50kD that was later confirmed as IgG H-chain (not shown) which provided an excellent contrast between the uniform reactivity towards IgA, and the inconsistent idiotypic reactivity of IgG resulting from variable sero-conversion to SET1. The exception was volunteer 12 (fig. 3B) who had no detectable SET1 reactive IgA and compensatory levels of anti-SET1 IgG. The

30   absence of IgA in individual 12 was later confirmed to be a result of a genetic IgA deficiency.

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Similar amounts of C5 were bound by SET1 from all sera although individuals 5 and 7 showed slightly lower reactivity that might reflect lower serum concentration of C5. C5 binding was clearly apparent in individual 12 confirming that SET1 binding to C5 was independent of IgA binding. This was later confirmed by Biosensor binding analysis (see below).

Analyses were performed on sera from other species and compared to reactivity with staphylococcal protein A (SpA) bound IgG. SET1 strongly bound IgA from human, chimpanzee, baboon and pig sera. Weaker but still detectable binding was observed for horse and rat sera but no detectable binding was seen for cow, sheep, mouse, rabbit, goat IgA. In contrast, SpA bound IgG from the serum from all species (fig. 3C). SET1 bound polypeptides consistent with complement C5 from human, chimpanzee, baboon, pig, sheep, goat and rabbit serum where SET1 appeared highly selective (Fig 3C). This experiment took no account of the known differences in serum levels of IgA and C5 across species, but nonetheless provided initial evidence that SSL7 reactivity with both IgA and C5 was not limited to humans. The sizes and SDS migration under reducing and non-reducing conditions of polypeptides bound by SSL7 from other species are consistent with IgA and C5.

Affinity purification of secretory IgA from bovine or ovine colostrums and human breast milk

Species reactivity in those that showed poor binding of serum IgA was further tested using milk as a source of secretory IgA. Samples of diluted human breast milk (40 mg/ml), diluted bovine or ovine colostrums (also diluted to 40 mg/ml) were passed through a recombinant SET1 HPLC affinity column (fig 10). Bound protein was eluted using high pH giving a typical profile shown in figure 10. The single peak eluted with high pH was subjected to non-reducing and reducing SDS PAGE (displayed as an insert of Fig. 10). Under non-reducing conditions, a protein with an estimated MW of approximately ~400kD was routinely observed in the three species corresponding to the predicted size of the IgA dimer (2 x 170 kD IgA molecules plus a single 75 kD secretory chain along with the 15 kD

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J (joining) chain). Under reducing conditions this resolved into the secretory chain and the IgA heavy chain (the light chain and J chain were eluted from the bottom of this 7.5% SDS PAGE gel to better separate the high MW polypeptides). In the bovine sample, the SC was slightly lower in molecular weight than the human and ovine SC, presumed to be due to

5   either differences in proteolytic processing or alternatively altered SC glycosylation between these species. The 75 kD and 60kD polypeptides were confirmed as SC and IgA Heavy chain respectively by peptide mass fingerprinting.

SET1 has nanomolar affinity for both human IgA and C5.

10 Biosensor analysis (BIAcore biosensor) confirmed binding to both IgA and C5 and established the affinity of both interactions. Recombinant SET1 protein was reacted with immobilized serum IgA (Calbiochem) and global analysis of the kinetic data fitted well ($chi^2 = 0.184$) to a model of parallel binding to a heterogeneous ligand with $K_{D1} = 1.0$ nM and for the second site $K_{D2} = 330$ nM (fig 4A). The fit was poor to other models including

15 a single site model. The random coupling of ligand by carbodiimide chemistry to a biosensor chip can result in some orientations of ligand unfavorable for interaction with the analyte. Thus the high affinity site, $K_{D1} = 1.0$ nM, most probably reflected the IgA:SET1 interaction. Since serum IgA consists of both IgA1 and IgA2 subclasses this also may contribute to the heterogeneity of the binding behaviour observed. Equilibrium binding

20 analysis, like the kinetic data, best fitted a two-site binding model yielding K_{D1} and K_{D2} values of 1.1 ± 0.2 nM and 80 ± 40 nM respectively for the high and low affinity sites (fig. 4B). Thus both the kinetic and the equilibrium analysis concur that SET1 displays nanomolar affinity for serum IgA. The binding to recombinant baculovirus produced IgA1-Fc region and to recombinant IgA2 (Gift of Drs Margaret Goodall and Roy Jefferies)

25 confirmed that SET1 binds to both IgA1 and IgA2 isotypes in the Fc region of the molecule (data not shown).

The interaction of SET1 with human C5 was also analyzed using the biosensor. SET1 protein was injected over the immobilized IgA followed by an injection of purified human

30 C5 (Sigma) to demonstrate C5 binding to the SET1 captured on IgA (fig 4C). This simultaneous binding of IgA and C5 to SET1 indicates these interactions occur through

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distinct and functionally separate sites, consistent with the serum binding studies that showed C5 from some species (e.g. rabbit) showed binding to SET1 independent of binding to IgA (fig 3C) and volunteer 12 who's complement C5 bound to SET1 in the absence of serum IgA.

5

The affinity of the SET1 interaction with human C5 was ascertained using IgA to capture SET1, which was then reacted with C5 in the analyte. In this case kinetic analysis was not possible because of the dissociation of the captured SET1 from the IgA layer. However equilibrium analysis was possible with the assumption that the binding of C5 to SET1 did

10   not affect the rate of dissociation of SET1 from the IgA layer. The data obtained fitted well to a single binding site, as often occurs with orientated presentation of ligands to analyte on the biosensor, with a $K_D = 18 \pm 1$ nM (fig 4D).

15

Allelic differences in SET1 do not alter IgA or C5 binding.

Some SETs such as SET1 exhibit considerable allelic variation among strains of *S. aureus*. To date, 5 alleles of the *SET1* gene have been published (MW2, NCTC8325, N315,

20   NCTC6571, Mu50). The amino acid sequences of these published alleles vary by 16% (fig. 5A). Two further alleles of *SET1* were obtained from local clinical isolates called GL1 and GL10. These were cloned and sequenced and compared for their ability to bind human serum IgA. SET1-GL1 and SET1-GL10 differ by 30/210 residues; a variation of 14% yet both bound similar amounts of human IgA from identical volumes of serum from two

25   different people (fig. 5B). Thus the IgA binding was not significantly altered by the 14% allelic variation observed between the GL1 and GL10 alleles.

SET1 blocks IgA binding to myeloid cells.

The receptor for IgA is FcαRI (CD89) and is expressed on myeloid cells such as

30   neutrophils, granulocytes, eosinophils, monocytes and macrophages (28). Flow cytometric analysis (FACS) of human granulocytes with FITC-serum polyclonal IgA was performed to determine whether SET1 blocked cell surface binding of IgA to FcαRI (CD89)

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expressed on myeloid cells such as neutrophils, granulocytes, eosinophils, monocytes and macrophages (28). SET1 was incubated at serial concentrations with a fixed concentration of FITC-IgA (10 μg) prior to incubation with freshly prepared human leukocytes. The superantigen, staphylococcal enterotoxin A (SEA), was used as a negative control. Dose

5   dependent inhibition of IgA binding to cells was observed with SET1 but not with SEA. 90% inhibition was achieved with a 10-fold (w/w) excess of SET1 (100 μg) over FITC-IgA (10 μg). This was equivalent to 70-fold molar excess (Figure 6). SEA had no inhibitory effect on FITC-IgA binding (Fig. 6). This suggested that SET1 most likely bound to a site on IgA overlapping the FcαR1 binding site in the Cα2:Cα3 domains of the

10   IgA H-chain (17).

SET1 blocks binding of IgA to purified FcαRI

To confirm SET1 inhibits of IgA binding to FcαRI, biosensor studies were performed using the FcαRI ectodomains expressed using recombinant baculovirus infected Sf21

15   insect cells. Purified recombinant soluble FcαRI was immobilized at a level of 500RU on a BIAcore CM5 biosensor chip. No binding of SET1 (200nM) to the FcαRI was observed (Fig. 7A). In the absence of SET1 binding of the 100nM serum IgA to the FcαRI was observed (~220 RU) with kinetics as reported previously (fig. 7B). Prior incubation of IgA with recombinant SET1 inhibited the binding of IgA to immobilized FcαRI. An

20   approximately equimolar concentration of SET1 (100nM) to IgA (100nM) reduced the receptor binding by 70% (62 RU, Figure 7C). Thus SET1 inhibited IgA binding to FcαRI both in its cell surface form and as soluble proteins with consistent inhibitory kinetics.

SET1 inhibits complement-mediated lysis and plasma bactericidal activity.

25   Serum from normal individuals was tested for naturally occurring complement lysis of allogeneic human RBC. Two sera IB and JL were found that readily lysed allogeneic RBC in a standard *in vitro* complement hemolytic assay in the presence of EGTA. IB serum was significantly more hemolytic (100% hemolysis at 2-fold dilution) than JL (40% hemolysis at 2-fold dilution). Hemolysis was negligible following serum heat inactivation

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(56°C for 30 minutes) or addition of EDTA but was unaffected by EGTA confirming the activation of the alternate complement pathway (not shown).

5   Addition of recombinant SET1 to the serum prior to the addition of erythrocytes had a profound inhibitory effect on both IB and JL serum initiated hemolysis (Fig. 8). Maximum inhibition by SET1 was obtained at 1.0 nM (0.63 µg/25 µl serum) for donor IB and 0.26 nM (0.16 µg/25 µl serum) for donor JL.   Interestingly, the approximately 3-fold lower amount of SET1 was required to inhibit JL lysis correlated with the 3-fold lower lytic activity of JL serum.   As a control, purified recombinant SEA that binds neither serum

10   IgA nor complement C5, displayed no inhibition even at the highest concentration tested. The slightly lower maximum lysis of IB serum between the SET1 inhibition experiment (~80%) and the SEA inhibition experiment (~50%) was most likely due to aging of the serum as these two assays were performed two days apart.

15   *SSL7 enhances survival of bacteria in serum.*
SET1 was tested for its ability to provide protection to a bacterium that was sensitive to plasma bactericidal killing. Recombinant SET1 was incubated with fresh human serum over a range of concentrations prior to the addition of ~10^7 E. coli K12 bacteria, a bacterium which does not survive in human blood, as it is susceptible to complement lysis.

20   In the absence of SET1, no cells survived after a 90-minute incubation of 10^7 bacteria at 37°C with 500 µl fresh serum diluted 1:2 in HBSS. Addition of recombinant SET1 at concentrations above 0.26 nM (7 µg/ml) profoundly inhibited serum anti-microbial activity and increased the number of surviving E. coli cells to a maximum level of 14% at 2.1 nM (50 µg/ml) (fig. 9). The control recombinant SEA had no effect on lysis even at the

25   highest concentration used (50 µg/ml).

Discussion

Herein describes the first reported function(s) for a member of the eleven gene cluster of SETs (or SSLs) located on the staphylococcal pathogenicity island, SaPIn2 ((3), (10)).

30   SET1 (or SSL7) selectively bound both purified IgA and complement C5 and inhibited complement and IgA:FcαRI binding - two important components of the host protective

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immune mechanisms against *S. aureus*. SET1 also inhibited the bactericidal activity of plasma against a sensitive organism (in this case *E. coli*) at concentrations consistent with the approximate serum concentration of complement C5. The structural homology between SETs and the superantigens argues a close evolutionary relationship between these protein families, but this study reveals that the prototypical SET family member, SET1, has unique functions not shared with the superantigen family. These functions, leukocyte IgA receptor inhibition and complement inhibition, mediated through binding IgA and C5 respectively, reflect specialization of this superantigen-like class in microbial defense. A number of other recombinant SETs have been tested for IgA binding and/or complement inhibition but so far, only SET1 displays this capacity.

SET1 bound with high affinity to IgA and inhibited IgA:FcαRI complex formation most probably through competition for binding at the IgA-Fc Cα2-Cα3 interface where the first ectodomain of FcαRI binds (17, 20-22, 26). SET1 inhibition of IgA binding to FcαRI was seen in a biosensor assay configured for high affinity bivalent capture of IgA (17, 26) and 70% inhibition was achieved by 1:1 addition of 100nM SET1 with 100nM IgA. The high affinity ($K_D \sim 1$ nM) binding of SET1 to each IgA would result in about 3/4s of the IgA molecules having one or both heavy chains occupied by SET1 and so unavailable for bivalent capture by immobilized rsFcαRI. Moreover, SET1 bound equally sIgA and momomeric IgA from serum, indicating that the SET1 could affect immune evasion both in humoral and mucosal environments. Competition of SET1 and rsFcαRI for binding to IgA suggests that the most likely scenario is two SET1 binding sites per IgA-Fc. Furthermore SET1 binding of secretory IgA suggests that either the SET1 and SC binding site are completely separate, or the single SC in the sIgA incompletely occupies the SET1 binding sites.

SET1 is the first example of a staphylococcal immunoglobulin binding protein that selectively targets IgA and provides a mode of defense not previously described for this pathogen. Staphylococcal protein A (SpA) is the prototypic staphylococcal immunoglobulin binding protein. It is a surface bound protein that binds predominantly to the Fc region of IgG through its repeat domains. This direct coating of the bacteria in IgG

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is thought to prevent IgG function by orientating the Fc in such a manner so that it cannot activate FcγRI or complement. In contrast to SpA, SET1 is a secreted protein apparently without multiple repeated binding domains that selectively targets an isotype not previously considered important in defense against staphylococci.

5

The ability of SET1 to block IgA binding to its FcαRI (CD89) suggests a possible role in inhibiting Fc receptor-mediated leukocyte activation that leads to respiratory burst, cytokine release and phagocytosis. The human pathogens *Streptococcus pyogenes* and group B streptococcus also produce IgA binding proteins suggesting there is a selective

10 advantage for these microbes to evade IgA-mediated defense mechanisms. These proteins, Arp4 and Sir22, are M proteins from *S. pyogenes* and the unrelated β protein from group B streptococcus all bind IgA-Fc at the same site as the leukocyte receptor FcαRI, and like SET1, also block IgA binding to FcαRI bearing granulocytes (26).

15 The binding and inhibition of complement C5 by SET1 is novel. The site of binding on C5 remains to be identified. One possible site would be the C3-convertase cleavage site preventing the formation of C5a anaphylatoxin and C5b initiator of MAC (preliminary evidence of binding of SET1 to activated C5 suggests however that it binds to the C5 beta chain – not shown). Here, SET1 was shown to inhibit complement-mediated hemolysis

20 and enhance bacterial survival of *E.coli* at concentrations similar to the concentration of C5 in serum. The inhibition of complement C5 cleavage to C5a anaphylatoxin would be a more important mechanism to the survival of S. aureus. C5a initiates inflammatory responses, leukocyte recruitment and phagocytosis, which are all important for the destruction of gram-positive organisms.

25

SET1 enhanced microbial survival, inhibiting plasma killing of *E. coli*, an organism that is sensitive to human plasma. *E. coli* was used instead of *S. aureus*, because the latter organism is likely to produce its own endogenous SET1 and is thus capable of better surviving in plasma without additional SET1. Inhibitory activity of this cytolytic activity

30 was first observed at concentrations above 0.3nM and increased to a maximum of approximately 1.7nM. This concentration is most consistent with the approximated

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concentration of C5 in serum (0.4nM). Inhibition was not profound with only a maximum of 0.03% surviving colonies (3000 colonies from an initial 10^7 inoculum). This would suggest *E. coli* is killed by a number of mechanisms, only one of which (C5 mediated complement) is inhibited by SET1. Notably, the plasma used was not depleted of

5 phagocytic cells that might represent an additional killing mechanism of *E. coli* not inhibited by SET1. SET1 likely represents only a component of a defense mechanism involving multiple factors including other members of the SET (or SSL) cluster.

The results described herein indicate SET1 affinity chromatography may offer a simple

10 single step method for the purification of IgA and C5, which have both traditionally been laborious molecules to isolate using classical methods.

The invention has been described herein with reference to certain preferred embodiments, in order to enable the reader to practice the invention without undue experimentation.

15 Those skilled in the art will appreciate that the invention is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. Furthermore, titles, headings, or the like are provided to enhance the reader's comprehension of this document, and should not be read as limiting the scope of the present invention.

20

The entire disclosures of all applications, patents and publications, cited above and below, if any, are hereby incorporated by reference.

The reference to any prior art in this specification is not, and should not be taken as, an

25 acknowledgment, or any form of suggestion, that that prior art forms part of the common general knowledge in the field of endeavour to which the invention relates in any country.

Throughout this specification, and any claims which follow, unless the context requires otherwise, the words "comprise", "comprising" and the like, are to be construed in an

30 inclusive sense as opposed to an exclusive sense, that is to say, in the sense of "including, but not limited to".

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